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Award Number: W81XWH-07-1-0301

TITLE: A Model System to Investigate the Effect of BRCAl and/or p53 Inactivation in the Ovarian Stroma on Growth and Transformation Potential of the Ovarian Epithelium

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REPORT DATE: May 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2008	Annual	1 APR 2007-30 APR 2008

4. TITLE AND SUBTITLE

A Model System to Investigate the Effect of BRCAl andor p53 Inactivation in the Ovarian Stroma on Growth and Transformation Potential of the Ovarian Epithelium

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0301

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fox Chase Cancer Center
Philadelphia, PA 19111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The tumor microenvironment plays an important role in cancer progression. The tumor stroma promotes angiogenesis and is a source of growth factors, chemokincs, and extracellular matrix (ECM) rnoleculcs that promotes carcinoma progression. We are investigating the effects of loss of function of BRCAl and Trp53 in the stroma on the growth and neoplastic transformation of epithclial cells using 2-D and 3-D in vitro culture models. Wamcn carrying gerrnline mutations in BRCAl are at high risk for developing ovarian cancer and mutations in pS3 arc frequently detected in ovarim tumors. Further, inactivation of BRCAl in mouse granulosa cells results in the development of benign epithelial neoplasm in the ovary and uterine horn, but the lesions themselves express wild-type BRCAl. Inactivation of BRCAl also causes arrest at the G21M transition and significantly increases population doubling times; an effect that is reversed by inactivation of Trp53. 'Therefore, to directly test the hypothesis that stromal cells excrt a ccll-nonautonomous effect on ovarian epithelial cell growth and neoplastic transformation, we will examine the effects of culturing mouse ovarian surface epithelial (MOSE) cells with ECM and conditioned media from stromal calls in which BRCAl and Trp53 have been inactivated (alone or in combination).

15. SUBJECT TERMS
BRCA 1, p53, ovarian cancer, epithelial/stromal - interaction

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

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A Model System to Investigate the effect of *BRCA1* and/or *p53* Inactivation in the Ovarian Stroma on Growth and Transformation Potential of the Ovarian Epithelium

PI: Denise C. Connolly
Progress Report, April 16, 2008

Introduction:

The tumor microenvironment, including the surrounding stroma, plays an important role in cancer progression. The tumor stroma facilitates angiogenesis and is a source of growth factors, chemokines and extracellular matrix (ECM) molecules that promote tumor progression. We are investigating the effects of loss of function of *BRCA1* and *Trp53* gene inactivation in the stroma on the growth and neoplastic transformation of epithelial cells using both 2-D and 3-D *in vitro* culture models. Women carrying germline mutations in *BRCA1* are at high risk for developing ovarian cancer (1) and mutations in *p53* are frequently detected in ovarian tumors (2). Interestingly, conditional inactivation of *BRCA1* in mouse granulosa cells results in the development of benign epithelial neoplasms in the ovary and uterine horn, but the lesions themselves express wild-type *BRCA1* (3). Inactivation of *BRCA1* also causes growth arrest at the G2/M transition and results in significantly increased population doubling times; an effect that is reversed by inactivation of *Trp53* (4). Therefore, to test the hypothesis that mutations in stromal cells exert cell-nonautonomous effects on ovarian epithelial cell growth and neoplastic transformation, we are studying the consequences of culturing mouse ovarian surface epithelial (MOSE) cells with ECM and conditioned media from stromal cells which harbor conditionally expressed mutant alleles of *BRCA1* and *Trp53*. MOSE and stromal cells are isolated from mice bearing loxP flanked (floxed) alleles of *BRCA1* and *Trp53* that are subsequently inactivated by infection with recombinant adenovirus containing the *Cre* recombinase gene.

The specific aims are:

1. Identify paracrine/autocrine factors from MOSE and stromal cells in which *BRCA1* and/or *Trp53* have been inactivated that influence pre-neoplastic/neoplastic changes in epithelia.
2. Assess the role that 3-D matrices (isolated from inactivated stromal cells, e.g., those with excised *Trp53*) play in the growth and transformation of MOSE cells with inactivated *BRCA1*.

Body:

The following goals were completed this year: 1) optimization of techniques for isolation and propagation of MOSE and stromal cells, 2) characterization of the purity of the isolated primary cell populations, and 3) optimization of adenoviral infection of cells to excise *BRCA1* and *Trp53*. All procedures described involving animals were approved by Fox Chase Institutional Animal Care and Use Committee.

Analysis of epithelial/stromal interactions that promote ovarian oncogenesis will ultimately be performed by culturing primary MOSE cells on a 3-D ECM extracted from primary stromal cells. MOSE and stromal cells were isolated from mice (4-6 months) of the following genotypes: 1) $BRCA1^{LoxP/LoxP}$ 2) $Trp53^{LoxP/LoxP}$, 3) $BRCA1^{LoxP/LoxP}$;$Trp53^{LoxP/LoxP}$, 4) $BRCA1^{LoxP/LoxP}$;$Trp53^{R172H/+}$ and 5) $BRCA1^{LoxP/LoxP}$;$Trp^{53R172H/LoxP}$. Bright field images of early passage (≤p2) MOSE are shown in **Figure 1**, demonstrating the successful isolation cells from each genotype. To assess the purity of MOSE and stromal cell isolations, expression of epithelial (E-cadherin, Cytokeratin 19 [CK19]) and mesenchymal (vimentin and smooth muscle actin [SMA]) markers were evaluated by indirect immunofluorescence. Immunolocalization of CK19 and SMA in MOSE cells isolated from $BRCA1^{LoxP/LoxP}$;$Trp53^{R172H/+}$ mice is shown in **Figure 2** and demonstrates that the cell population predominantly expresses CK19 and little or no detectable levels of SMA. A similar analysis of epithelial and mesenchymal marker expression in the stromal cell cultures was also performed. The stromal cell population isolated from $BRCA1^{LoxP/LoxP}$;$Trp53^{R172H/+}$ mice exhibits high levels of both vimentin and SMA immunoreactivity (shown in **Figure 3**), but no expression of the epithelial marker, E-cadherin, which has been

previously shown to be expressed in normal MOSE cells (4). These results confirm our capability to successfully isolate pure populations of ovarian epithelial and stromal cells.

To test our hypothesis that the stroma plays a cell-nonautonoumous role in promoting ovarian oncogenesis requires conditional inactivation of *BRCA1* and/or *Trp53*. In this system, conditional inactivation of the *BRCA1* and/or *Trp53* alleles requires expression of the Cre-recombinase to mediate excision of floxed sequences. For our experiments we have chosen to infect purified epithelial and stromal cell populations with an adenoviral vector containing both *Cre* recombinase and a GFP reporter (*Ad5-CMV-Cre-GFP*) to monitor infection efficiency. Cells infected with an adenoviral vector containing only the *GFP* reporter serve as controls. Infection efficiency of the adenoviral vector, *Ad5-CMV-GFP*, was evaluated in both epithelial and stromal cells. Adenoviral infection of MOSE cells isolated from *BRCA1*$^{LoxP/LoxP}$;*Trp53*$^{R172H/+}$ mice (**Figure 4**) was performed as previously described (5) and expression of the GFP reporter was visualized 72 h post-infection. The results show that the adenovirus efficiently infects MOSE cells. Infection of mouse ovarian stromal cells by adenovirus *in vitro* has not been reported. Therefore, adenovirus infection was optimized by treating stromal cells isolated from *BRCA1*$^{LoxP/LoxP}$;*Trp53*$^{R172H/+}$ mice with adenovirus at increasing multiplicities of infection (MOIs). Stromal cells expressing the GFP reporter are shown in **Figure 5.** These results show that infection efficiency increases with higher MOIs. Although the infection efficiencies for both MOSE and stromal cells appear to be high, further enrichment of infected cells (GFP-expressing) can be accomplished by fluorescent activated cell sorting (FACS) as nesessary. An alternative strategy is to perform a second viral infection to increase the efficiency of Cre-mediated excision.

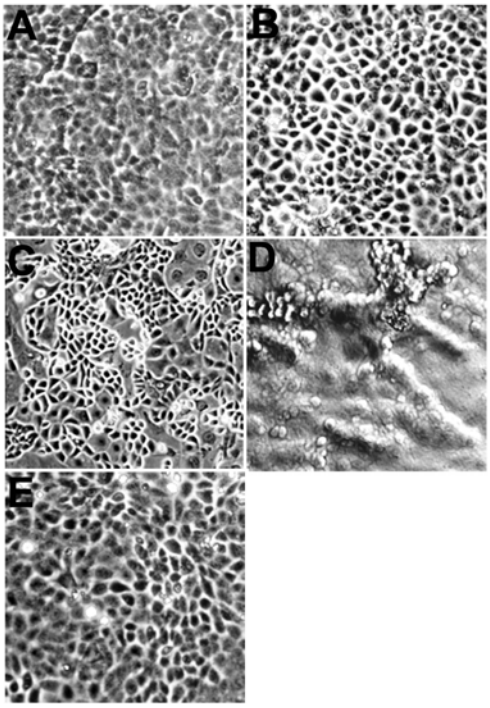

Figure 1. **Early passage MOSE cells**. Bright field images of MOSE cells isolated from A) *BRCA1*$^{LoxP/LoxP}$ (p1) , B) *Trp53*$^{LoxP/LoxP}$ (p2), C) *BRCA1*$^{LoxP/LoxP}$;*Trp53*$^{LoxP/LoxP}$ (p2), D) *BRCA1*$^{LoxP/LoxP}$;*Trp53*$^{R172H/+}$ (p1) and E) *BRCA1*$^{LoxP/LoxP}$;*Trp*$^{53R172H/LoxP}$ (p1) mice. All images are at 10X magnification and the passage number is indicated in parenthesis.

MOSE cells and stromal cells isolated from all five genotypes either have been or are currently undergoing *Ad5-CMV-Cre-GFP* infection to mediate excision of floxed alleles of *BRCA1* and/or *Trp53*. MOSE cells isolated from mice that constitutively express a mutant *p53* allele (*BRCA1*$^{LoxP/LoxP}$;*Trp53*$^{R172H/+}$ and *BRCA1* $^{LoxP/LoxP}$;*Trp*$^{53R172H/LoxP}$) grow more robustly in primary culture and therefore have been the most easily isolated and expanded. These cells were the first to undergo Ad-Cre-GFP infection. Cre recombinase-mediated excision of floxed alleles of *BRCA1* and/or *Trp53* is verified by PCR amplification of genomic DNA using the same methods we use to genotype mice. Once excision is confirmed, stocks of all cell lines are frozen and cultures are propagated to collect conditioned media. In addition, wild type and mutant (*BRCA1* and/or

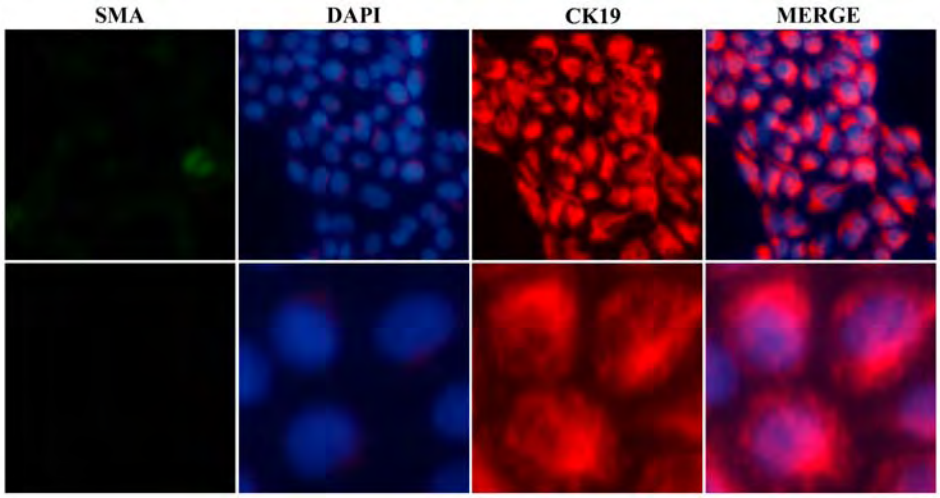

Figure 2. Expression of epithelial and mesenchymal markers in MOSE cells. MOSE cells isolated from *BRCA1*$^{LoxP/LoxP}$;*Trp53*$^{R172H/+}$ mice were stained with antibodies to SMA (green) and Cytokeratin 19 (CK19/Troma III; red) and detected by indirect immunofluorescence at low (10X, top row) and high (40X, bottom row) magnification. Nuclei were stained with DAPI.

Trp53 excised) stromal cell cultures will be grown as 3-D cultures for extraction of ECM from these cells as previously described (6). MOSE cells will then be cultured on the 3-D matrix. This will allow us to evaluate the contribution of: 1) *BRCA1*, 2) *Trp53*, 3) *BRCA1* and *Trp53*, and 4) the mutant $Trp53^{R172H}$ on growth and apoptosis as well as other tumorigenic functions such as migration and invasion. MOSE cells have a long doubling time *in vitro* and doubling time varies with genetic background. Population doubling time for each of the five genotypes will be determined and compared to wild type MOSE cells. When sufficient numbers of MOSE cells of the remaining genotypes ($BRCA1^{LoxP/LoxP}$, $Trp53^{LoxP/LoxP}$ and $BRCA1^{LoxP/LoxP}$;$Trp53^{LoxP/LoxP}$) have been isolated, Ad-Cre-GFP mediated infection will be performed to evaluate epithelial/stromal interactions in the various *BRCA1* and *Trp53* genetic backgrounds.

MOSE and stromal cells isolated from the five different genotypes described above is shown in **Table 1**. Both frozen stocks and isolations that are currently in culture are included in this table. We have been able to isolate stromal cells and MOSE cells from all of the genotypes which are either frozen or currently in culture.

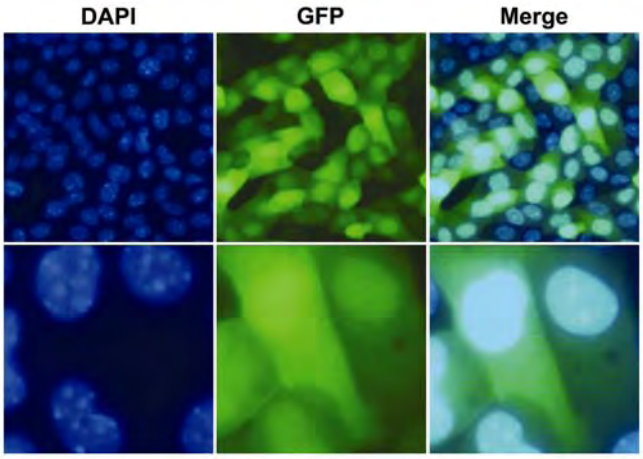

Figure 4. Adenoviral infection of MOSE cells. MOSE cells isolated from $BRCA1^{LoxP/LoxP}$;$Trp53^{R172H/+}$ mice were infected with Ad5-CMV-GFP at a MOI of 200 for 72 h. Low (10X) and high (40X) power magnification of immunofluorescent images showing GFP expression. Nuclei were counterstained with DAPI.

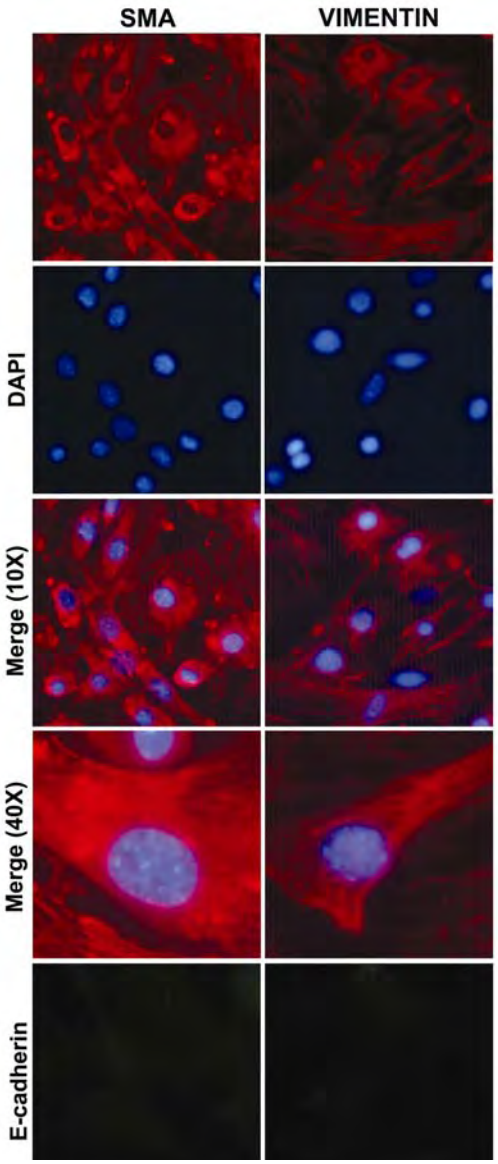

Figure 3. Expression of epithelial and mesenchymal markers in stromal cells. Stromal cells isolated from $BRCA1^{LoxP/LoxP}$;$Trp53^{R172H/+}$ mice were stained with A) Vimentin (red) and SMA (red) immunolocalization at 10X magnification. B) Vimentin and SMA staining at 40X magnification (top row). E-cadherin (green) expression (bottom row) in the same cells stained with anti-vimentin and anti-SMA. Nuclei were counterstained with DAPI.

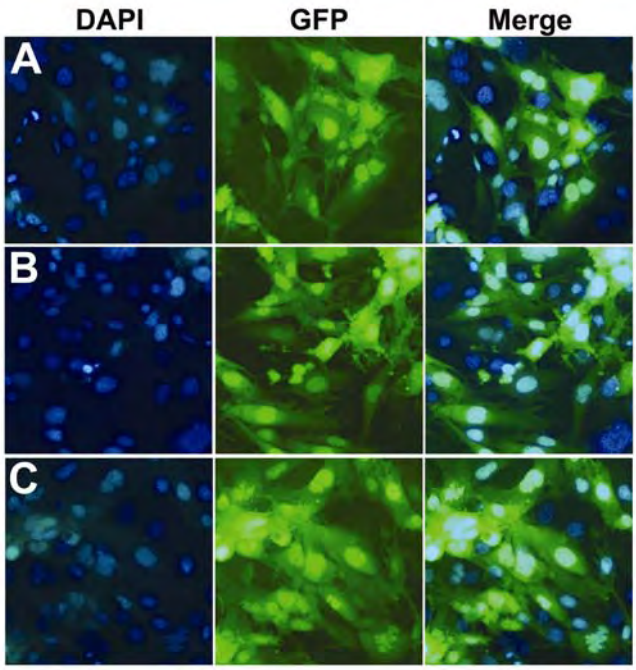

Figure 5. Adenoviral infection of stromal cells. Stromal cells isolated from *BRCA1*$^{LoxP/LoxP}$*;Trp53*$^{R172H/+}$ mice were infected with Ad5-CMV-GFP at MOI of A) 200, B) 400, and C) 800 for 72 h. Immunofluorescent images at 10X magnification show GFP expression in stromal cells. Nuclei were counterstained with DAPI.

Table 1. Epithelial and stromal primary cell stocks

Genotype	MOSE Stocks	Stromal Stocks	Cells in Culture
BRCA1$^{LoxP/LoxP}$	--	P4 (4/1/08), 5 vials P4 (4/14/08), 5 vials	P2 (3/31/08), MOSE P5 (4/8/08), MOSE P4 (4/14/08), stromal
Trp53$^{LoxP/LoxP}$	--	P7 (2/20/08), 8 vials	P2 (3/31/08), stromal P3 (4/8/08), stromal
BRCA1$^{LoxP/LoxP}$***;Trp53***$^{LoxP/LoxP}$	--	P5 (12/21/07), 7 vials P5 (3/13/08), 13 vials P5 (3/1/08), 8 vials	P7 (4/9/08), MOSE
BRCA1$^{LoxP/LoxP}$***;Trp53***$^{R172H/+}$	P5 (12/21/07), 3 vials P7 (2/20/08), 4 vials P8 (3/31/08), 6 vials	P6 (2/20/08), 10 vials P7 (3/08), 6 vials	P3 (4/8/08), MOSE
BRCA1 $^{LoxP/LoxP}$***;Trp***$^{53R172H/LoxP}$	P7 (3/21/08), 2 vials	P4 (11/30/07), 17 vials P3 (12/21/07), 4 vials	N/A

Key Research Accomplishments:

- Isolation and propagation of primary MOSE and stromal cells which will allow analysis of stromal/epithelial interactions from 5 different *BRCA1* and *Trp53* genetic backgrounds
- Isolation of mouse cancer stromal cells and control, wild-type stromal cells
- Characterization of isolated primary cells with epithelial and mesenchymal markers by indirect immunofluorescence
- Establishment of frozen stocks of primary cells
- Efficient adenoviral infection of primary cells

Reportable Outcomes:

- MOSE cells preferentially grow in small colonies on top of stromal cells
- MOSE cells carrying the *Trp53*^{R172H} allele have a growth advantage over cells with wild-type *Trp53*
- MOSE cells carrying the *Trp53*^{R172H} allele form spheroids or groups of cells that detach from the monolayer and grow in suspension (see **Figure 1D**)

Conclusions:

We are able to isolate and propagate primary MOSE and stromal cells from mice bearing floxed alleles of *BRCA1* and *Trp53*. Cultured MOSE and stromal cells can be infected at high efficiency with recombinant adenovirus constructs harboring GFP and Cre-recombinase-GFP. The availability of primary MOSE and stromal cell cultures allows us to investigate the role of epithelial/stromal interactions that may contribute to the growth of OSE cells and the development of preneoplastic/neoplastic changes. Our experiments are focused on the potential significance of expression of mutant forms *BRCA1* and *Trp53* genes in epithelial/stromal interactions. These reagents will be important for studying the early steps that initiate ovarian tumor formation within the context of a more relevant 3-D culture model, and addresses the roles of cell-nonautonomous functions contributed by the stromal microenvironment.

Appendices:

Appendix A – Mice utilized for MOSE and stromal cell isolations

References:

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Appendix A- Mice used in isolation of epithelial and stromal cells

Table 1. ***BRCA1$^{LoxP/LoxP}$***

Mouse #	DOB[a]	DOD[b]	MOSE STOCKS	STROMAL STOCKS
522	2/20/07	10/5/07		
523	2/20/07	10/5/07		
524	2/20/07	10/5/07		
525	2/20/07	10/5/07		
530	2/21/07	10/5/07	First isolation. No cells.	N/A
531	2/21/07	10/5/07		
533	2/21/07	10/5/07		
537	3/15/07	10/5/07		
538	3/15/07	10/5/07		
539	3/15/07	10/5/07		
555	4/19/07	10/19/07		
556	4/19/07	10/19/07		
557	4/19/07	10/19/07	P7 (1/31/08), 1 vial, not viable	Not viable
558	4/19/07	10/19/07		
559	4/19/07	10/19/07		
653	9/22/07	2/19/08		
656	10/4/07	2/19/08		
661	10/4/07	2/19/08		
664	10/4/07	2/19/08		
666	10/4/07	2/19/08	P5 (4/8/08), in culture	P4 (4/1/08), 5 vials
667	10/4/07	2/19/08		
668	10/4/07	2/19/08		
669	10/4/07	2/19/08		
674	10/3/07	2/19/08		
675	10/3/07	2/19/08		
681	10/31/07	3/11/08		
682	10/31/07	3/11/08		
683	10/31/07	3/11/08	P2 (3/31/08), in culture	P4 (4/14/08)
684	10/31/07	3/11/08		
690	10/31/07	3/11/08		
691	10/31/07	3/11/08		

[a]DOB = date of birth
[b]DOD = date of death

Table 2. ***Trp53$^{LoxP/LoxP}$***

Mouse #	DOB[a]	DOD[b]	MOSE STOCKS	STROMAL STOCKS
239	5/15/07	10/12/07		
240	5/15/07	10/12/07		
242	5/9/07	10/12/07		
243	5/9/07	10/12/07		
247	5/20/07	10/12/07	P7 (1/30/08), 1 vial, not viable	N/A
248	5/20/07	10/12/07		
249	5/20/07	10/12/07		
250	5/20/07	10/12/07		
251	5/20/07	10/12/07		
828	6/19/07	12/19/07		
829	6/19/07	12/19/07		
830	6/19/07	12/19/07	P3 (4/8/08), in culture	P7 (2/20/08), 8 vials
832	6/19/07	12/19/07		
264	7/02/07	12/19/07		

272	10/4/07	3/26/08		
274	10/26/07	3/26/08		
275	11/10/07	3/26/08		
276	11/10/07	3/26/08	P2 (3/31/08), in culture	P3 (4/2/08), in culture
277	11/10/07	3/26/08		
278	11/10/07	3/26/08		
279	11/10/07	3/26/08		

[a]DOB = date of birth
[b]DOD = date of death

Table 3. ***BRCA1$^{LoxP/LoxP}$;Trp53$^{LoxP/LoxP}$***

Mouse #	DOB[a]	DOD[b]	MOSE STOCKS	STROMAL STOCKS
772	3/18/07	10/9/07		
773	3/18/07	10/9/07		
781	3/14/07	10/9/07		
782	3/14/07	10/9/07		
783	3/14/07	10/9/07	Poor proliferation	N/A
784	3/14/07	10/9/07		
785	3/14/07	10/9/07		
800	5/16/07	10/9/07		
791	5/15/07	11/15/07		
792	5/15/07	11/15/07		
793	5/15/07	11/15/07		
796	5/16/07	11/15/07	P8 (2/1/08), 1 vial, not viable	P5 (12/21/07), 7 vials
797	5/16/07	11/15/07		
812	6/10/07	11/15/07		
819	6/19/07	11/15/07		
820	6/19/07	12/19/07		
823	6/19/07	12/19/07		
828	7/11/07	12/19/07		P5 (1/28/08), 5 vials
829	7/11/07	12/19/07	Not viable	Not viable
830	7/11/07	12/19/07		
836	7/11/07	12/19/07		
837	7/11/07	12/19/07		
849	9/7/07	02/25/08		
850	9/7/07	02/25/08		
851	9/7/07	02/25/08		
859	10/2/07	02/25/08	P7 (4/9/08), in culture	P5 (3/13/08), 13 vials
860	10/2/07	02/25/08		
862	10/2/07	02/25/08		
863	10/2/07	02/25/08		
861	10/2/07	03/06/08		
858	10/2/07	03/06/08	Not viable	P5 (3/31/08), 8 vials
877	10/2/07	03/06/08		
879	10/2/07	03/06/08		

[a]DOB = date of birth
[b]DOD = date of death

Table 4. ***BRCA1$^{LoxP/LoxP}$;Trp53$^{R172H/+}$***

Mouse #	DOB[a]	DOD[b]	MOSE STOCKS	STROMAL STOCKS
1033	4/17/07	10/19/07		
1035	4/17/07	10/19/07		
1036	4/17/07	10/19/07	P5 (12/21/07), 3 vials	P6 (12/4/07), 10 vials
1049	5/07/07	10/19/07		
1061	6/17/07	12/17/07		
1063	6/17/07	12/17/07		
1064	6/17/07	12/17/07		
1067	6/14/07	12/17/07	Not viable	P6 (2/20/08), 10 vials
1068	6/14/07	12/17/07		
1069	6/14/07	12/17/07		
1072	6/14/07	12/17/07		
1073	6/14/07	12/17/07		
1094	8/3/07	12/21/07		
1095	8/3/07	12/21/07		
1118	8/23/07	12/21/07	P7 (2/20/08), 4 vials,	P7 (3/08), 6 vials
1119	8/23/07	12/21/07	P8 (3/31/08), 6 vials	
1120	8/23/07	12/21/07		
1128	8/30/07	12/21/07		
1151	10/8/07	3/26/08		
1144	10/11/07	3/26/08		
1188	10/31/07	3/26/08	P3 (4/8/08), in culture	P4 (4/8/08), in culture
1180	11/01/07	3/26/08		
1183	11/10/07	3/26/08		

[a]DOB = date of birth
[b]DOD = date of death

Table 5. ***BRCA1$^{LoxP/LoxP}$;Trp$^{53R172H/LoxP}$***

Mouse #	DOB[a]	DOD[b]	MOSE STOCKS	STROMAL STOCKS
114	5/1/07	10/30/07		
115	5/1/07	10/30/07		
116	5/23/07	10/30/07		
117	5/23/07	10/30/07	Poor proliferation	P4 (11/30/07), 17 vials
118	5/23/07	10/30/07		
119	5/23/07	10/30/07		
120	5/23/07	10/30/07		
121	5/23/07	11/8/07		
122	5/23/07	11/8/07		
123	5/23/07	11/8/07		
124	5/23/07	11/8/07		
125	5/23/07	11/8/07	P7 (3/21/08), 2 vials	P3 (12/21/07), 4 vials
126	5/23/07	11/8/07		
127	5/23/07	11/8/07		
128	5/23/07	11/8/07		
129	5/23/07	11/8/07		

[a]DOB = date of birth
[b]DOD = date of death